



Variation of apoptotic and proliferative activity among lymphoma subtypes in dogs: A flow cytometric study

Fulvio Riondato^{a,*}, Valeria Martini^b, Maverick Melega^a, Alessia Poggi^a, Barbara Miniscalco^a

^a Department of Veterinary Sciences, University of Turin, largo Braccini 2-5, 10095 Grugliasco, Turin, Italy

^b Department of Veterinary Medicine, University of Milan, via dell'Università 6, 26900 Lodi, Italy

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ABSTRACT

Tumor growth depends on both proliferative and apoptotic rate of neoplastic cells. High proliferation index is a well-known negative prognostic factor in canine lymphomas, whereas little is known about apoptotic activity. We describe proliferative and apoptotic rates in different canine lymphoma subtypes at diagnosis. Flow cytometry (FC) was used to assess the percentage of proliferating cells (Ki67%) and of apoptotic cells (AnnV%) in 128 lymph node (LN) aspirates from dogs with lymphoma. Proliferation/apoptosis ratio (PAR) and turnover index (TI; Ki67% + AnnV%) were then calculated for each case. High-grade B-cell lymphomas showed high values for both Ki67% and AnnV%, low-grade B-cell lymphomas showed low Ki67% and high AnnV%, high-grade T-cell lymphomas showed high Ki67% and low AnnV%, and low-grade T-cell lymphomas showed low levels of both parameters. Lymphoblastic lymphomas had the highest PAR values. High-grade B-cell lymphomas had the highest TI values while small clear cells lymphomas the lowest. The panorama of proliferative and apoptotic activity widely varies among lymphoma subtypes. Our results lay the ground for future clinical and pharmacological studies.

1. Introduction

Proliferative rate is one of the key factors for subtype classification and prediction of prognosis in dogs with lymphoma (Aresu et al., 2015; Miniscalco et al., 2018; Poggi et al., 2015; Ponce et al., 2004; Valli et al., 2013). Cellular proliferation in canine lymphomas has been evaluated by means of several techniques including morphological examination alone (Fournel-Fleury et al., 1997a; Greenlee et al., 1990; Teske et al., 1994), immunocytochemistry (Fournel-Fleury et al., 1997b), immunohistochemistry (Bauer et al., 2007; Kiupel et al., 1999; Phillips et al., 2000), and flow cytometry (FC) (Miniscalco et al., 2018; Poggi et al., 2015).

Tumor growth depends on both cell proliferation and amount of cell loss, which is mostly related to the apoptotic rate. However, only few studies reported apoptotic rates in clinical samples of canine lymphoma. About twenty years ago, Phillips and colleagues reported a high intra-subtype variability of proliferative and apoptotic rate in canine lymphomas (Phillips et al., 2000). These parameters were coupled into two indexes: the proliferation/apoptosis ratio (PAR), which could predict relapse-free interval (RFI), and the turnover index (TI, sum of proliferative and apoptotic fractions). The authors do not provide data on single lymphoma subtypes, nor at-

tempt any comparison among histotypes. Also, the low number of cases and the inclusion of different lymphoma subtypes in the survival analyses are major limitations of the study.

More recently, Meichner and colleagues documented a higher Bcl-2/Bax ratio in T-cell than in B-cell lymphomas and non-lymphomatous lymph nodes (LN) (Meichner et al., 2016). Both molecules are involved in the control of apoptotic activity, with Bcl-2 having pro-survival, and Bax pro-apoptotic activity. Thus, a higher ratio is suggestive of enhanced resistance to apoptosis in T-cell lymphomas.

The results obtained in the studies by Phillips and Meichner suggest that inhibition of apoptosis may play a role in the pathogenesis and clinical behavior of canine lymphomas, with differences among subtypes. Furthermore, induction of apoptosis is a key-event in chemotherapeutic treatment of lymphoma in dogs (Pawlak et al., 2014; Pawlak et al., 2017). Deepening the knowledge of the apoptotic rate of each lymphoma subtype before treatment may prove useful in order to select the most appropriate therapeutic regimen for each case.

The aim of the present study was to depict the panorama of proliferative and apoptotic rates in canine lymphomas and to highlight possible differences among subtypes.

* Corresponding author.

E-mail address: fulvio.riondato@unito.it (F. Riondato)

2. Materials and methods

2.1. Case selection

Samples included in the study were selected among lymph node aspirates submitted to the FC service of the Veterinary Teaching Hospital of the University of Turin (IT) between January 2017 and December 2019. Cases were included if fulfilling the following criteria: 1) final diagnosis of nodal lymphoma based on clinical presentation, complete blood count, and cytological and FC analysis of an enlarged peripheral LN; 2) availability of a good quality LN cytological smear; 3) LN aspirate cellularity sufficient to assess proliferation and apoptotic rates by FC. Patients treated with corticosteroids or chemotherapy prior to FC analysis were excluded. All dogs were privately owned and sampled for diagnostic purposes with an informed consent of the owners. Thus, according to the authors' institution guidelines, a specific approval to use the leftover specimens for research purposes was not required (EC Decision nr 1965/2017, University of Turin).

2.2. Classification of lymphomas

Cytological preparations were reviewed by the same observer and classified according to the updated Kiel classification (Fournel-Fleury et al., 1997a, 1997b). Lymphoma phenotype was determined on LN aspirates as previously described (Miniscalco et al., 2018), using the antibody panel reported in Table 1. Samples were collected into RPMI tubes, kept refrigerated and processed within 24 h. All samples were acquired with a BD Accuri C6 flow cytometer (Becton Dickinson) and analyzed with the specific software CFlow Plus (Becton Dickinson). The operator dealing with cytological specimens was aware of the lymphoma phenotype detected by FC. Because of the low prevalence of some Kiel subtypes, cases were categorized according to their phenotype and grade in four groups: high-grade B-cell, low-grade B-cell, high-grade T-cell and low-grade T-cell (Miniscalco et al., 2018).

2.3. Assessment of proliferation and apoptotic rate

Proliferation rate was defined as the percentage of Ki67+ cells out of total nucleated cells (Ki67%). Briefly, an intracytoplasmic labelling with a FITC-conjugated anti-Ki67 monoclonal antibody (clone MIB-1, DAKO) was performed; a commercial permeabilization

kit (Leucoperm, Bio-Rad) was used according to manufacturer's instructions with an additional intermediate incubation step with frozen methanol (Poggi et al., 2015). A control tube was prepared in the same way except for the addition of FITC-conjugated isotypic immunoglobulin instead of the anti-Ki67 antibody.

Apoptotic rate was defined as the percentage of cells staining positive for Annexin V, but negative for Propidium Iodide (AnnV%). A commercial kit was used to this aim (AnnexinV-FITC, Apoptosis detection kit, eBioscience), according to the manufacturer's instructions. Briefly, cells were suspended in 200 μ l of Binding Buffer with a concentration of 2×10^5 cells/tube and incubated for 10 min at room temperature with 5 μ l of anti-AnnexinV-FITC antibody. Thereafter, 10 μ l of propidium iodide (PI) were added and incubated for 5 min before acquisition at the cytometer. A negative control with unstained cells was also acquired for each sample. Ki67% and AnnV% were finally coupled to calculate PAR (Ki67% / AnnV%) and TI (Ki67% + AnnV%).

2.4. Statistical analyses

Descriptive statistics were calculated for each continuous variable within each lymphoma subgroup. A Shapiro-Wilk test was performed to assess normal distribution of data. Thereafter, differences in Ki67%, AnnV% and PAR among the four lymphoma subgroups were evaluated using a Kruskal-Wallis test and a Mann-Whitney test for post-hoc analyses. Conversely, since TI data were normally distributed within each subgroup, differences in TI among the four lymphoma subgroups were evaluated using an ANOVA test and a Dunnett T3 test for post-hoc analyses.

Differences among Kiel subtypes were not investigated by means of statistical analysis since the low number of cases in less represented subtypes would have nullified statistical power of the analysis. Thus, only gross inspection of raw data was performed to describe the fluctuations of Ki67%, AnnV%, PAR and TI among Kiel subtypes and respective considerations are reported in the discussion.

All analyses were performed with a statistical software (SPSS v20.0 for Windows). Significance was set at $p \leq 0.05$ for all analyses, except for Mann-Whitney test, whereby it was set at $p \leq 0.008$ (based on the number of possible paired contrasts) in order to decrease the family-wise error rate.

3. Results

Overall, 128 lymphoma samples fulfilled our inclusion criteria. Breed was known for 123 dogs, including 82 (66.7%) pure breed and 41 (33.3%) mixed breed dogs. Sex was known for 124 dogs, including 63 (50.8%) females (30 spayed) and 61 (49.2%) males (8 neutered). Age was known for 118 dogs: mean age was 8.4 ± 3.2 years (median 8 years, min-max 1–15 years).

Table 2 reports the number of cases for each Kiel subtype and their respective Ki67%, AnnV%, PAR and TI values. Fig. 1 shows Ki67% and AnnV% of each case. Ninety (70.3%) dogs were diagnosed with B-cell and 38 (29.7%) with T-cell lymphoma; 104 (81.3%) dogs had a high-grade lymphoma and 24 (18.8%) had a low-grade lymphoma. Thus, 82 (64.1%) dogs had a high-grade B-cell lymphoma, 22 (17.2%) had a high-grade T-cell lymphoma, 16 (12.5%) had a low-grade T-cell lymphoma and 8 (6.3%) had a low-grade B-cell lymphoma.

Ki67%, AnnV%, PAR and TI for the four lymphoma subgroups are reported in Table 3. All parameters were significantly different among the four lymphoma subgroups ($p < 0.001$ for all analyses). Ki67% was significantly lower in low-grade T-cell lymphomas than in any other subgroup ($p < 0.003$ for all analyses) and in low-grade

Table 1
Antibody panel used for flow cytometric analysis of lymph node aspirates.

Target	Antibody clone	Fluorochrome	Reactivity
CD21	CA2.1D6	Alexa Fluor 647	Mature B-lymphocytes
CD79b(cy)	AT107-2	FITC	B-cells, including early precursors
CD79a(cy)	HM57	Alexa Fluor 647	B-cells, including early precursors
CD3	CA17.2A12	FITC	T-cells
CD3cy	CD3-12	FITC	T-cells
CD5	YKIX322.3	FITC	T-cells
CD4	YKIX302.9	Alexa Fluor 647	T-helper cells
CD8 α	YCATE55.9	PE	T-cytotoxic cells
CD45	YKIX716.13	FITC/PE/Alexa Fluor 647	All leukocytes
MHC II	YKIX334.2	FITC	Monocytes, Histiocytes, Lymphocytes
CD34	1H6	PE	Precursors

Table 2

Proliferative and apoptotic activity in 128 samples of canine nodal lymphomas, as calculated via flow cytometry, according to the cytological subtype of the neoplasia (updated Kiel classification).

Lymphoma subtype	Nr of cases	Ki67%	AnnV%	PAR	TI
		Mean \pm standard deviation (median, range)			
High-grade B-cell					
Centroblastic polymorphic	47	38.2 \pm 17.6 (36.1, 13.0–85.0)	69.8 \pm 16.7 (72.5, 30.0–94.4)	0.6 \pm 0.3 (0.5, 0.2–1.9)	107.9 \pm 25.5 (109.6, 58.7–166.6)
Centroblastic monomorphic	17	39.6 \pm 17.0 (37.0, 7.8–70.2)	71.0 \pm 16.3 (74.6, 30.6–92.8)	0.6 \pm 0.4 (0.6, 0.1–1.7)	110.6 \pm 26.1 (114.9, 62.8–156.1)
Immunoblastic	11	18.6 \pm 5.0 (19.4, 10.0–26.7)	61.7 \pm 16.8 (64.9, 39.3–87.4)	0.3 \pm 0.1 (0.3, 0.1–0.5)	80.4 \pm 18.6 (82.0, 54.9–107.4)
Lymphoblastic	6	44.8 \pm 18.1 (40.6, 27.0–70.9)	38.3 \pm 25.5 (38.3, 10.2–72.8)	2.2 \pm 2.4 (1.1, 0.4–6.7)	83.1 \pm 28.2 (82.2, 37.2–121.8)
Plasmacytoid	1	13.0	61.1	0.21	74.1
Low-grade B-cell					
Macronucleolated medium-sized	6	7.7 \pm 3.2 (8.1, 3.3–12.0)	57.6 \pm 11.3 (56.2, 43.0–71.9)	0.1 \pm 0.1 (0.1, 0.1–0.2)	65.3 \pm 11.7 (65.7, 46.3–77.5)
Centrocytic	1	6.8	52.5	0.1	59.3
Prolymphocytic	1	9.0	60.0	0.2	69.0
High-grade T-cell					
Pleomorphic large	9	48.8 \pm 12.6 (46.0, 30.2–67.2)	17.9 \pm 6.5 (19.1, 9.8–28.8)	3.1 \pm 1.3 (2.9, 1.4–5.5)	66.8 \pm 14.6 (64.5, 47.5–86.3)
Pleomorphic mixed small and large	8	30.5 \pm 17.0 (25.8, 4.6–54.0)	20.7 \pm 19.7 (14.6, 4.2–52.6)	3.9 \pm 4.4 (2.2, 0.1–12.9)	51.2 \pm 17.2 (55.3, 27.4–78.2)
Lymphoblastic	5	42.5 \pm 10.8 (41.0, 28.9–58.1)	21.3 \pm 22.0 (11.1, 3.4–54.5)	5.7 \pm 5.1 (5.2, 0.5–12.1)	63.8 \pm 15.8 (69.2, 44.4–83.4)
Low-grade T-cell					
Small clear	14	2.9 \pm 1.1 (3.2, 1.3–4.2)	9.3 \pm 5.4 (9.1, 1.8–21.0)	0.5 \pm 0.5 (0.4, 0.1–2.1)	12.2 \pm 5.7 (11.9, 3.4–22.7)
Prolymphocytic	1	6.0	13.0	0.5	19.0
Pleomorphic small	1	11.4	5.4	2.1	16.8

Ki67%, percentage of cells staining positive for Ki67; AnnV%, percentage of cells staining positive for Annexin V but negative for Propidium Iodide; PAR, proliferation/apoptosis ratio (Ki67%/AnnV%); TI, turnover index (Ki67% + AnnV%).

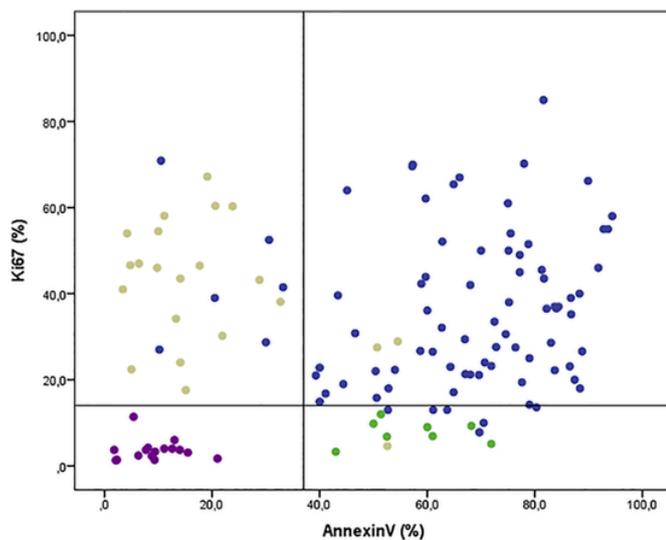


Fig. 1. proliferative and apoptotic rates in lymph node aspirates from 128 dogs with nodal lymphoma, assessed by flow cytometry. Blue dots: high-grade B-cell lymphomas. Green dots: low-grade B-cell lymphomas. Grey dots: high-grade T-cell lymphomas. Purple dots: low-grade T-cell lymphomas. Quadrants were divided arbitrarily according to the clouds of dots grossly discriminable. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

B-cell lymphomas than in high-grade B-cell and T-cell lymphomas ($p < 0.001$ for both analyses).

AnnV% was significantly higher in high-grade B-cell lymphomas than in high-grade and low-grade T-cell lymphomas ($p < 0.001$ for both analyses) and in low-grade B-cell lymphomas than in high-grade and low-grade T-cell lymphomas ($p < 0.001$ for both analyses). AnnV% did not significantly vary between high- and low- grade lymphomas of the same phenotype.

PAR was significantly higher in high-grade T-cell lymphomas than in any other lymphoma subtype ($p < 0.001$ for all analyses) and lower in low-grade B-cell lymphomas than in any other lymphoma subtype ($p < 0.001$ for all analyses). Nineteen out of 22 dogs (86.4%) with high-grade T-cell lymphoma had PAR > 1 (higher Ki67% than AnnV%) and only 3 among all of the 95 dogs (3.2%) with PAR < 1 had a high-grade T-cell lymphoma.

TI was significantly higher in high-grade B-cell lymphomas than in any other lymphoma subtype ($p < 0.001$ for all analyses) and lower in low-grade T-cell lymphomas than in any other lymphoma subtype ($p < 0.001$ for all analyses), whereas difference was not significant between low-grade B-cell and high-grade T-cell lymphomas ($p = 0.929$). Forty-five out of 82 dogs (54.8%) with high-grade B-cell lymphoma had a TI $> 100\%$, while all the cases in other subgroups had TI values lower than 87%.

4. Discussion

Proliferation rate is a well-known malignancy index in canine lymphomas, whereas apoptotic rate has been far less studied. Still, both phenomena contribute to tumor growth. In the present study,

Table 3

Proliferative and apoptotic activity in 128 samples of canine nodal lymphomas, as calculated via flow cytometry, according to the grade and phenotype of the neoplasia.

Lymphoma subgroup	Nr of cases	Ki67%	AnnV%	PAR	TI
		Mean \pm standard deviation (median, range)			
High-grade B-cell	82	36.0 \pm 17.7 (32.8, 7.8–85.0)	66.5 \pm 19.0 (69.9, 10.2–94.4)	0.7 \pm 0.8 (0.5, 0.1–6.8)	102.6 \pm 27.0 (104.0, 37.2–166.6)
Low-grade B-cell	8	7.8 \pm 2.8 (8.0, 3.3–12.0)	57.3 \pm 9.8 (56.3, 43.0–71.9)	0.1 \pm 0.1 (0.1, 0.1–0.2)	65.0 \pm 10.3 (65.7, 46.3–77.5)
High-grade T-cell	22	40.7 \pm 15.8 (43.4, 4.6–67.2)	19.7 \pm 15.5 (14.6, 3.4–54.5)	4.0 \pm 3.6 (2.8, 0.1–12.9)	60.4 \pm 16.7 (57.9, 27.4–86.3)
Low-grade T-cell	16	3.6 \pm 2.4 (3.5, 1.3–11.4)	9.3 \pm 5.2 (9.1, 1.8–21.0)	0.6 \pm 0.6 (0.4, 0.1–2.1)	12.9 \pm 5.7 (12.5, 3.4–22.7)

Ki67%, percentage of cells staining positive for Ki67; AnnV%, percentage of cells staining positive for Annexin V but negative for Propidium Iodide; PAR, proliferation/apoptosis ratio (Ki67%/AnnV%); TI, turnover index (Ki67% + AnnV%).

we report proliferation (Ki67%) and apoptotic (AnnV%) rates, as well as their ratio (PAR) and sum (TI), in different canine lymphoma subtypes at diagnosis.

As expected, major differences in Ki67% were found between high-grade and low-grade lymphomas in our case series. Conversely, mean AnnV% was higher in B-cell than in T-cell lymphomas, irrespective of the grade. These results allowed the delineation of the four previously defined lymphoma groups based on patterns of proliferation and apoptosis (Fig. 1): high-grade B-cell lymphomas showed high values for both parameters, low-grade B-cell lymphoma showed low Ki67% and high AnnV%, high-grade T-cell lymphomas showed high Ki67% and low AnnV%, and low-grade T-cell lymphomas showed low levels of both parameters.

High-grade T-cell lymphomas had the highest mean PAR value, as a result of a vigorous proliferative activity combined with scant apoptotic rates. Our results are consistent with those obtained few years ago by Meichner and colleagues, who revealed higher Bcl-2/Bax ratio in T-cell than in B-cell canine lymphomas (Meichner et al., 2016). Resistance to apoptosis seems to be similarly enhanced in low-grade T-cell lymphomas, based on the low AnnV% values recorded in our case series. However, in this subgroup of dogs, the small percentage of apoptotic cells is balanced by slow proliferation rates, resulting in intermediate PAR values.

High-grade B-cell lymphomas also demonstrated intermediate PAR values. However, in this subset the result was related to simultaneous high proliferation and apoptosis rates. Conversely, TI values of high-grade B-cell lymphomas were about 10 fold higher than low-grade T-cell lymphomas, demonstrating a higher cellular turnover in the former subgroup. This accelerated turnover in high-grade B-cell lymphomas is associated with a rapid spread of the disease and onset of clinical signs. Importantly, the reduced (virtually null) percentage of quiescent cells in high-grade B-cell lymphomas is a favorable issue for affected dogs, since chemotherapeutic drugs act exclusively on cycling cells: indeed, dogs with high-grade B-cell lymphomas have the highest probability to obtain complete remission after treatment (Aresu et al., 2015). In contrast, most cells in low-grade T-cell lymphomas are quiescent, resulting in a slow, progressive accumulation of neoplastic cells in the organisms that can even remain unnoticed from a clinical point of view. Only few proliferating cells counteract the high apoptotic rates in low-grade B-cell lymphomas, resulting in the lowest PAR values in our case series and accounting for low aggressiveness and less responsiveness to standard chemotherapeutic protocols of these entities.

Interestingly, more than half high-grade B-cell lymphoma had TI >100%. This implies the presence of apoptotic cells staining positive for Ki67, or proliferating cells undergoing apoptosis. Unfortunately,

the lack of a multicolor FC approach for Ki67 staining in our study prevented us from determining the percentage of Ki67 + AnnV+ double positive cells. Still, this observation suggests the presence of a proliferation-associated apoptosis. It is not surprising, since entering cell cycle is considered a crucial step toward apoptosis (Rubbin et al., 1993). However, our data support the existence of proliferation-independent apoptosis in canine lymphomas too, since the percentage of cells staining positive for Annexin V exceeded those staining positive for Ki67 in B-cell lymphomas. This phenomenon has already been documented in human neutrophils (Coates et al., 1996). Further studies are needed to assess the molecular link between proliferation and apoptosis in canine lymphoma cells.

Beyond lymphoma subgroups, some interesting observations arise from data on Kiel subtypes. High-grade B-cell lymphomas displayed variable proliferative and apoptotic rates. If compared to centroblastic lymphomas (both polymorphic and monomorphic), immunoblastic lymphomas had slightly lower PAR due to lower Ki67%, while lymphoblastic lymphomas had higher PAR due to much lower AnnV%. The lower PAR and Ki67% values observed in immunoblastic lymphomas may be a consequence of a cytological misclassification of some macronucleolated medium size cells (MMC) lymphomas (low-grade) in this group. In fact, immunoblastic lymphomas are composed of a dominant population of immunoblasts characterized by large size, single large nucleolus and extended hyperbasophilic cytoplasm (Fournel-Fleury et al., 1997a, 1997b) that closely resemble the macronucleolated cells of MMC.

Also, the possible transformation from low to high grade between B-cell lymphoma subtypes (Franz et al., 2013; Comazzi et al., 2015), makes their cytological discrimination more challenging. Additionally, MMC (marginal-zone) lymphoma is currently classified as a low grade malignancy based on morphological parameters. Still, recent findings support that this lymphoma subtype shares underlying pathogenetic mechanisms with the centroblastic (diffuse large B-cell) lymphoma subtype (Richards et al., 2013). Similarly, clinical behavior of marginal-zone lymphoma reflects the one of diffuse large B-cell lymphomas (Cozzi et al., 2018), limiting the clinical usefulness of the discrimination between these two entities. Further prospective studies are warranted to determine whether PAR or TI may contribute in predicting B-cell lymphomas prognosis in dogs, irrespective of morphological classification.

Lymphoblastic lymphomas had the highest PAR values among both B-cell and T-cell lymphomas. This result may be linked to the aggressive behavior of this type of lymphoma. Lymphoblastic lymphoma arises from precursor cells and forms a unique entity with the acute lymphoblastic leukemia according to the World Health Organization classification scheme adapted to canine hematopoietic neo-

plasms (Valli et al., 2011). It is characterized by an extremely poor prognosis in dogs, even poorer than other high-grade lymphomas of the same phenotype (Aresu et al., 2015; Novacco et al., 2015; Ponce et al., 2004).

On the other side, small clear cells lymphomas show the lowest AnnV% among all Kiel subtypes. Resistance to apoptosis coexists with only minimal proliferative activity, as documented by the low Ki67% detected (consistently <5%). As stated above, this combination of phenomena (low proliferation and resistance to apoptosis), which seems to be peculiar of this specific lymphoma subtype, may cause a slow accumulation of neoplastic cells in the organism and may explain the indolent nature of the disease and prolonged survival of affected dogs. Small clear cells (T-zone) lymphoma also shows specific peculiarities from a biological and clinical point of view, including phenotypic aberrancies, consistent advanced stage at diagnosis, gross blood and minimal marrow infiltration (Martini et al., 2015; Martini et al., 2016; Seelig et al., 2014). The data we present in the present study further support the distinctiveness of this lymphoma subtype.

The major limitation of this study is the lack of non-lymphomatous samples. In particular, data on proliferative and apoptotic rates of normal and reactive lymph nodes may serve as a control to better understand the biology of low-grade lymphomas. Secondly, the low number of some Kiel subtypes with low prevalence in dogs, prevented any specific consideration. Thirdly, a multicolor approach with contextual determination of AnnV and Ki67 would have strengthened the suggestion about the presence of both proliferation-independent and proliferation-associated apoptosis. Finally, most of the included cases do not have a histopathological diagnosis. However, the combination of cytology and FC is considered an accurate substitute to confirm the diagnosis of lymphoma and define grade and phenotype of the neoplasm (Poggi et al., 2015; Rout and Avery, 2017).

In conclusion, the present study provides data on proliferative and apoptotic activity of lymphomatous cells in dogs. Our results deepen the knowledge of the pathobiology of the different lymphoma subtypes highlighting similarities and differences, and may serve as a basis for future pharmacological studies on the activity of chemotherapeutic drugs on lymphoma cells in dogs. Further studies should also address possible prognostic role of PAR and TI within specific lymphoma subtypes.

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